9. Details of experimental design and procedures - Continued

well known, but it is difficult to conceive how a blood HbCO of eg. 5% in a smoker, might result in a visual decrement due to the slight diminution of blood oxygen carrying capacity, and slight shift of the HbO_2 dissociation curve to the left, which this low level might cause. Lilienthal (1950) has pointed out that it is at least theoretically possible for CO to exert some of its effects via binding to tissue cytochromes, but this is generally dismissed as improbable due to insufficient blood concentrations of dissolved CO even at severe levels of CO intoxication (see, eg, discussion by LaPresle and Fardeau, 1967). This latter view, however, neglects the possibility for active transport mechanisms for CO into tissues as has in fact been shown to occur in lung and placenta (Burns and Gurtner, 1968) mediated by cytochrome p-450 which apparantly functions as a carrier for both CO and O_2 from blood to tissues. Also, in our own laboratory we (Ingenito et al, 1974) have demonstrated a long-lasting negative ionotropic action of CO on the perfused isovolumic rabbit heart with a hemoglobin free perfusate. This study established that CO can be carried into tissues from a dissolved state and can be bound to some myocardial tissue component, possibly myoglobin or the cytochromes. That a CO-cytochrome complex might be responsible for the effects of CO in the visual system is given further impetus from the statement of Halperin et al.,(1959) "The results described (for CO and the visual system)... appear to point definitely to the presence, somewhere in the visual and/or nervous systems of some hitherto unrecognized substance with hematin-like properties and of marked physiological importance in vision." The presence and importance of such a substance was recently demonstrated by Shichi (1969) who found a cytochrome p-450 in the microsomal frac-

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9. Details of experimental design and procedures - Continued

of tobacco smoke. We submit that it is time to give the visual system some detailed consideration.

Department of Ophthalmology at Albany Medical College and occupies laboratory space in the Pharmacology Department, is an expert on electroretinography, has published studies on this subject (Rabin and Berson, 1974) and has indicated a willingness to help set-up and run the instrumentation for electroretinography in the Basic Science

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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 12/30/74

JAN 20 1975

1. Principal Investigator (give title and degrees):

Alphonse J. Ingenito, Ph.D.
Associate Professor of Pharmacology

2. Institution & address:

The Albany Medical College of Union University
Albany, New York 12208

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

Actions of Carbon Monoxide and Tobacco Smoke on Retinal Metabolism and Function

5. Proposed starting date: July 1, 1975.

6. Estimated time to complete: Two years.

7. Brief description of specific research aims:

The project to be described will assess, in the acute experimental situation, the effects of tobacco smoke and carbon monoxide (CO) at concentrations comparable to those occurring in tobacco smoke, on retinal metabolism and function. The main aim will be to determine whether the deficits in visual function reported to occur following inhalation of small concentrations of CO or heavy tobacco smoking, might have their origin in the retina, specifically on certain CO-sensitive biochemical functions of the pigment epithelium of retina (to be described below). Using both in vitro and in vivo physiological and biochemical systems in bovine, porcine, feline and rabbit eyes, an attempt will be made to correlate any pharmacological effects observed on retinal function with those observed on biochemical processes. An attempt will also be made to relate the data to the possibility of deficits in visual function caused by heavy smoking and exposure to environmental levels of CO.

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9. Details of experimental design and procedures - Continued

tion of pigment epithelial cells in bovine retina. Shichi showed that this CO-sensitive cytochrome functioned as part of an electron-transport system which catalyzed the oxidation of NADPH and NADH and he suggested that it might function in the retina to effect retinol-retinaldehyde interconversion, an important component of the rhodopsin cycle in visual cells. (Wald 1968.) The pigment eptihelium is considered to be of prime importance in mediating the exchange of blood borne nutrients and gases between the choroidal circulation and the rods and cones and to carry out essential metabolic and supporting functions for the visual cells. (Bernstein 1960, Porter and Yamada 1960, Moyer 1969). Its destruction by natural causes (retinitis pigmentosa) or by chemical agents (Potts, 1965) can lead to blindness.

Experimental Design and Procedures

The protocol to be described will attempt to correlate biochemical effects in an in-vitro system with possible pharmacological actions of CO and tobacco smoke on in-vivo preparations. The key hypotheses to be tested are: (1) that an effect of CO and/or tobacco smoke on visual function can be defined at the level of the retina itself using acute, in-vivo and in-vitro preparations and (2) that an effect of CO and/or tobacco smoke can be described on a cytochrome-containing enzyme system in pigment epithelium which converts retinol to retinaldehyde.

I. Electrophysiological Studies

To establish actions on the retina for CO and/or tobacco smoke, three preparations are contemplated:

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Budget Justification

A. Salaries

The principal investigator plans to spend approximately 50% of his time on this research project over the next two years, if the grant is awarded. The amount of salary requested, however, ($5,000) amounts to only 25% of his present salary. This is in order to keep salary requests to a reasonable figure. From the literature provided us by the Tobacco Research Council, we are not aware of any particular policy on salaries for professional workers. The amount requested for a full-time technician ($9,000) is a figure consistent with Albany Medical College's institutional policy as suggested by the College's Director of Personnel, for an individual holding a Bachelor's degree in Chemistry or Biology. The projected budget for the second year contains a $500 increase in salary for this individual, as acknowledgement for increased experience and proficiency.

B. Consumable Supplies

This category is difficult to itemize in detail, but the figure of $4,500 is one which we find from previous experience to be an approximate amount for a new project where new types of glassware and chemical reagents need to be ordered. The chromatographic separation of Vitamin A aldehyde, alcohol and esters will require some new equipment for column and thin-layer chromatography which we do not presently have on hand and we feel that the figures given are a reasonable estimate of what will be required for the first year.

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9. Details of experimental design and procedures - Continued

1. Electroretinogram of the Anesthesized Cat:

We plan to monitor the electroretinogram (ERG) of the anesthetized cat (pentobarbitol or chloralose, depending upon which causes less interference with ERG recordings) made to inhale various concentrations of CO in oxygen or air, so as to allow arterial blood HbCO levels to rise to "smoking levels" (between 5 and 15%). The ERG will be evoked by photic stimulation (Grass photostimulator). The results will be compared with exposures to similar degrees of hypoxia induced with nitrogen so as to establish whether the effects are secondary to hypoxia or are due instead to some other action of CO. Cats will also be made to inhale 25 ml puffs of tobacco smoke from University of Kentucky research cigarettes and the ERG monitored. The ERG is considered to be a good indicator of retinal function and its various components have been intensely investigated as to their origins in discrete layers of the retina (Davson 1972, Brown, 1968, Brown and Wiesel, 1959, Jacobson 1961, Crescitelli 1960, Noell 1954 and many others). The c-wave of the ERG has particular importance for the proposed study since its origin is said to be the pigment epithelium and it is depressed by toxic agents such as sodium azide, and sodium iodate which selectively destroy pigment epithelium. (Noell, 1954; Potts, 1965) The existing literature (op cit above and others) on anesthetic and other drug effects on the ERG will serve as a guide in the interpretation of the ERG. The recording of the ERG is a relatively simple and non-invasive technique which preserves the functional status of retina and pigment epithelium. We will most likely be able to use our existing equipment for this with the exception of some corneal and other electrodes and a

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III. PROFESSIONAL EXPERIENCE (cont'd):

nitrogen) on brain and cardiac metabolism and function. Effects of carbon monoxide on the metabolism and function of the visual system (retina, optic nerve and brain).

IV. OTHER PROFESSIONAL EXPERIENCE:

Registered Pharmacist, states of New York and New Jersey.
Employed as a retail pharmacist in New Jersey, 1956 to 1958.

V. SERVICE WITH ARMED FORCES:

U. S. Army, October 1954 to August 1956.
Highest rank attained - private first class.
Occupational duty - hospital pharmacist with evacuation hospital in Korea.
Separation - July 1956. Honorable Discharge - 1962.

VI. PROFESSIONAL AFFILIATIONS:

Member - American Society for Pharmacology and Experimental Therapeutics. since 1970
Committee on Public Affairs, 1973-76
Fellow - American College of Clinical Pharmacology, since 1971
Program Chairman, Annual Meetings, 1973 and 1974
Member - AAAS
Vice President, New York State Society for Medical Research

VII. MARITAL AND DEPENDENCY STATUS:

Married (1962) with 4 children.

Wife: Barbara Joan Steffen
Children and year of birth: Diana Lynn (1963), Jeanne Marie (1964), Carol Ann (1966), James Francis (1973).

VIII. PUBLICATIONS:

Ingenito, A. J. and Bonnycastle, D. D.: Amine Levels in Rat Brain During Cold Acclimation and Following Reserpine and Acute Cold Exposure. The Pharmacologist 6: 194 (1964) (abstract).

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VIII. PUBLICATIONS (cont'd):

Ingenito, A. J. and Bonnycastle, D. D.: The Effect of Exposure to Heat and Cold Upon Rat Brain Catecholamines and 5-Hydroxytryptamine Levels. Can. J. Physiol. Pharmacol. 45: 733-743 (1967).

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Barrett, J. P., Ingenito, A. J. and Procita, L.: A Brain Perfusion Technique Adapted for the Study of Drugs Which May Affect the Peripheral Circulation Through a Central Action. J. Pharmacol. Exp. Therap. 170: 199-209 (1969).

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9. Details of experimental design and procedures - Continued

as described by Glocklin and Potts (1962) and incubating these, in suitable buffers equilbrated with CO, smoke or N_2 mixtures or tobacco smoke, in the presence of added quantities of retinol, in order to study the conversion of retinol to retinaldehyde both in the medium and in the cells. Retinaldehyde will be determined by the colormetric method of Futterman and Saslow (1961) and retinol by the colormetric method of Futterman (1963). If inhibition is detected with CO or tobacco smoke, we will attempt to determine the lowest concentration of CO or aqueous tobacco smoke extracts necessary to cause a measurable inhibition of retinaldehyde to retinol interconversion. Shichi (1969) makes no mention of the requirements for the presence or absence of light in his microsomal assay system so this aspect will need to be investigated. It would appear, offhand, that the reaction would proceed best in the dark, as it does in the natural state.

If time allows, we will also make some comparative studies, in the dark, of the ability of bovineor porcine retinal visual cell outer segments prepared according to Futterman (1963) or McConnell (1965) to conduct the same reaction, since all authorities do not agree that retinol-retinaldehyde conversion takes place only in pigment epithelium (Bridges, 1970).

The above evaluations will be carried out either ina Warburg type apparatus (Gilson Respirometer) which we have on hand or, more simply in a Dubnoff metabolic shaker, which will need to be purchased, but which is quicker to use and will allow more determinations in the same run.

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In the second technique for studying pigment epithelium biochemistry,

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VIII. PUBLICATIONS (cont'd):

Ingenito, A. J., Barrett, J. P. and Procita, L.: An Analysis of The Effects of Nicotine on the Cerebral Circulation of an Isolated, Perfused In-Situ Cat Brain Preparation. STROKE-A Journal of the Cerebral Circulation 2: 67-75 (1971).

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Ingenito, A. J., Fiedler, P. C. and Procita, L.: Negative Inotropic Action of Carbon Monoxide on the Isolated Isovolumic Heart with a Hemoglobin-Free Perfusate. Fed. Proc. 33: 503 (1974) (abstract).

Ingenito, A.J., Ryon, J.J. and Procita, L.: Carbon Monoxide-Nicotine Interactions on the Isolated, Perfused, Isovolumic Rabbit Heart. Fed. Proc. 1975 (abstract in the press).

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Budget Justification

C. Other Expenses

We estimate that our studies on the cat electroretinogram (ERG) will require between 50 and 75 cats and our metabolic studies on rabbit retina will require about 100 rabbits. Present cost of cats are about $16.00 and for rabbits about $6.00 each, totaling about $1200 for the former and $600 for the latter, leaving about $200 of the total $2000 budgeted for animal care for the first year. A local abbatoir has agreed to supply us with freshly excised porcine eyes at 50 cents per eye. At least 50 eyes will be required weekly to obtain sufficient pigment epithelium to make once or twice weekly studies. If 30 weeks are allotted to these studies for the first year at $25.00 per week, the stated figure of $750 is arrived at.

Photographic and illustration work will be done by our Medical Illustrations Department of the College and will include graph drawing, ERG photograph development and mounting, picture taking and mounting for journal papers and 2 x 2 slide preparations for meeting presentations.

Computer analyses and consultation for statistical analyses of biochemical and physiological data may be required beyond the capabilities provided by our Department computer for routine analyses. We feel that the sum of $100 is a modest one for this eventuality.

The travel funds are requested so that the principal investigator may present papers at the annual meetings of FASEB and the Pharmacology Society (ASPET) each of which he is a member of.

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D. Permanent Equipment

The papers cited on the methods for studying cytochrome containing

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CURRICULUM VITAE

Alphonse J. Ingenito

The Albany Medical College of Union University
Albany, New York

(518) 445-5413

I. BIRTHPLACE AND DATE: Harrison, New Jersey; October 8, 1932

II. EDUCATION:

1. St. John's University, College of Pharmacy, New York, New York; B.S., 1953 (Pharmacy).
2. Rutgers University, College of Pharmacy, Newark, New Jersey; M.S., 1960 (Pharmacology).
3. University of Wisconsin Medical School,Dept. Pharmacology, Madison, Wisconsin; 1960-62; No degree granted.
4. College of Medicine and Dentistry of New Jersey at Newark, Dept. Pharmacology; Ph.D., 1965 (Pharmacology).

III. PROFESSIONAL EXPERIENCE:

1. Rutgers University, College of Pharmacy. Assistant Instructor in Biological Sciences, Sept. 1958 to June 1960. Laboratory instruction to undergraduate students in Pharmacology and other biological sciences. Research in Pharmacology leading to M.S. degree, concerned with CNS actions of antihistaminic-antiserotonin compounds.

2. University of Wisconsin Medical School, Dept. of Pharmacology. U.S.P.H.S. Pre-Doctoral Trainee, Sept. 1960 to July 1962. Research in Pharmacology in the psychopharmacology program concerned with the functional role of biogenic amines in developing rat and chicken brains.

3. College of Medicine and Dentistry of New Jersey at Newark, Dept. of Pharmacology. U.S.P.H.S. Pre-Doctoral Trainee, Sept. 1962 to July 1965. Lecturing and laboratory instruction to medical and dental students. Research in Pharmacology leading to Ph.D. degree concerned with the role of central biogenic amines in temperature regulation in the rat, and the interactions of various drugs with brain amines and body temperature.

4. Albany Medical College, Dept. of Pharmacology. Instructor in Pharmacology, July 1965 to July 1968. Assistant Professor of Pharmacology, July 1968 to July 1972. Associate Professor of Pharmacology, July 1972 to present. Lecturing and laboratory instruction in Pharmacology to medical students and other health sciences students and in graduate Ph.D. program in Pharmacology. Research with an in-situ brain perfusion technique in cats designed to assess the effects of drugs influencing the peripheral circulation by a central action. Effects of tobacco smoke constituents (nicotine, carbon monoxide, oxides of

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9. Details of experimental design and procedures - Continued

ready to collect data on the physiological aspects. It should be mentioned in this context that Mr. Arnold Rabin, presently a student in our second year medical class, and who is associated with the Department of Ophthalmology at Albany Medical College and occupies laboratory space in the Pharmacology Department, is an expert on electroretinography, has published studies on this subject (Rabin and Bersen, 1974) and has indicated a willingness to help set-up and run the instrumentation for electroretinography in the intact cat and perfused cat eye.

We could easily conceive that the above project might lead us to additional studies on related aspects of the smoking and vision problem, possibly involving clinical ERG studies with smoking humans, more detailed studies on retinal biochemistry and an analysis of the relative susceptibilities of different areas of the visual pathways to CO, a study somewhat analogous to the elegant analysis of the effects of anoxia on the various areas in the visual pathways by Noell and Chinn (1950). Also in view of our recent findings (Ingenito et al., 1975) that the effects of combinations of CO and nicotine in the rabbit heart differed from the effects of either agent alone, some studies on combinations of CO with other tobacco smoke constituents on the visual system would seem to be of importance.

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A casual glance through Larson et al.'s Tobacco monographs will convince the reader that the main emphasis of the tobacco and health problem has been placed on the effects of tobacco smoking on the cardiovascular and respiratory systems with relatively little emphasis on other systems. Perhaps it is time for some studies on those systems having a demonstrated sensitivity to certain of the active components

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An Analysis of the Effects of Nicotine on the Cerebral Circulation of an Isolated, Perfused, in situ Cat Brain Preparation

BY ALPHONSE J. INGENITO, PH.D., JAMES P. BARRETT, PH.D., M.D.,* AND LEONARD PROCITA, PH.D.

Abstract: *An Analysis of the Effects of Nicotine on the Cerebral Circulation of an Isolated, Perfused, in situ Cat Brain Preparation*

■ To determine the effects of nicotine HCl on the cerebral circulation of the cat, without the complicating actions of the drug at other sites in the body, the drug was perfused at concentrations of 1, 10 and 100 μg/ml through a vascularly isolated, perfused in situ cat brain preparation. Cats having selective section of cranial nerves 9, 10, 11 and 12 and the cervical sympathetic trunks comprised various experimental groups. Nicotine was also perfused through an isolated, denervated hindlimb of the same cat for comparative purposes. Nicotine caused only a mild and transient vasoconstriction of the cerebral circulation, mediated primarily by stimulation of the superior cervical ganglia, and a small direct cerebral vasoconstrictor component. An acute tolerance to the cerebral vasoconstrictor effects of repeated increments of nicotine was also observed. The cerebral vasoconstrictor effect of nicotine was diminished in the presence of intact vagi, suggesting a cerebral vasodilator role for these nerves. In contrast, the effect of nicotine on the denervated hindlimb vasculature was a weak but sustained vasodilation. The mechanism of action of nicotine on the cerebral circulation is discussed along with the relevance of these findings to the potential health hazards of tobacco smoking in individuals with cerebrovascular insufficiency.

ADDITIONAL KEY WORDS
innervation of cerebral circulation
smoking and cerebral ischemia
nicotine tolerance
cerebral vasoconstriction
vagal cerebral vasodilation
nicotine and hindlimb circulation

Introduction

□ Despite occasional speculation as to the relationship between cerebrovascular disease and smoking[1-3] and the suggestion that smoking be discouraged in patients with cerebral vascular insufficiency,[4] relatively little is known of the effects of smoking or nicotine on the cerebral circulation.[5, 6] Some studies in man have shown little or no effect of cigarette smoking or intravenously injected nicotine (in "smoking" doses) on cerebral blood flow and cerebrovascular resistance (CVR).[7-9] Larson et al.[5] cite a number of older studies on experimental animals in which cerebral blood flow was measured directly or by visual observation of exposed cortical vasculature following tobacco smoke inhalation or systemically injected nicotine. In most of these studies the action of nicotine was either vasodilator or

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*Present address: Department of Medicine, Germantown Hospital, Philadelphia, Pennsylvania.

Reprint requests should be sent to Dr. Alphonse J. Ingenito, Department of Pharmacology, Albany Medical College, Albany, New York, 12208.

Supported by a grant-in-aid from the American Medical Association Education and Research Foundation, Committee for Research on Tobacco and Health.

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9. Details of experimental design and procedures - Continued

glucose utilization and depletion of ATP stores in the medium and tissue. Alternatively, an action on some other transporting mechanism or carrier for O_2, between the medium and the tissue, might force the retina to anaerobic glycolysis, increased glucose utilization and increased lactate production. Lactate production is said to be especially critical in the retina because the acidity which it produces in the extracellular space may be of importance in maintaining a high tissue pO_2 in the relatively avascular retina via a Bohr shift in the HbO_2 dissocation curve, (Baeyens, et al., 1971). Pyruvate production will be studied as well since lactate/pyruvate ratios are considered to be a good indicator of the oxidative energy state of the tissue. ATP, glucose, lactate and pyruvate levels in both retinal tissue and incubation media will be done using established methods already being used in this laboratory. Since light might influence these reactions in the isolated retina, it will be necessary to conduct the incubations in both light and dark and in retinae taken from both light and dark adapted rabbits.

Under optimal conditions, the studies outlined above should take a full two years. We expect that during the first year considerable progress would be made on the biochemical aspects, since this is work with which we are familiar and have existing equipment to work with. During the first year we will need to have built, to assemble, and to test the equipment needed for the physiological studies on retina, including that for electroretinography and optic nerve potentials in the isolated retina-optic nerve preparation and the associated baths and perfusion devices. By the second year of the project we should be

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9. Details of experimental design and procedures - Continued

photostimulator which will need to be purchased. A Tektronix Dual beam Oscilloscope with Polaroid Camera or a Beckman Dynograph will be used to record the electrical potentials involved.

2. Isolated Perfused Cat Eye (Hemoglobin-Free Perfusate):

The above intact eye preparation, although important to establish the effects of CO and tobacco smoke on the relatively intact eye, presents some problems in interpretation because the effects of CO, however slight as they be, are employed. Consequently, it will be necessary to assess retinal function in a hemoglobin-free biological salt solution under controlled conditions. The optics of this system are rather complex and will need significant modification in our approach to its construction. The perfusion system (via the ophthalmic artery) is rather simple and should be adaptable

II. Biochemical Studies

The biochemical studies on retina will center around two techniques:

1. Studies on suspensions of and microsomal preparations of bovine and/or porcine pigment epithelial cells.

The studies will be designed to determine whether carbon monoxide, tobacco smoke, or aqueous extracts of tobacco smoke can inhibit the enzymatic conversion of retinol to retinaldehyde by the cytochrome p-450 microsomal oxidase system. This will be done in two ways: In the first method, we will prepare suspensions of pigment epithelial cells

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9. Details of experimental design and procedures - Continued

we plan some limited work using Shichi's (1969) method for preparing microsomal fractions of pigment epithelial cells, in which the enzyme preparation is obtained in more concentrated form, allowing a study of the associated reduction of NADH or NADPH, this being an integral part of the oxidation of retinol. Changes in the spectrum caused by conversion of oxidized to reduced pyridine nucleotides may be determined in the spectrophotometer. This technique will also allow us to examine the spectral characteristics of the cytochrome p-450 containing enzyme in suspension. If the p-450 fraction is involved in the inhibition there should be bands of spectral absorbtion detectable at 447 and 470 mμ (Shichi, 1969). With some precautions taken to maintain a CO-atmosphere during the transfer of incubation mixture to cuvette, we should be able to detect evidence of CO-hemeprotein complexes in our microsomal reaction mixtures. This should constitute prima facie evidence of CO-induced enzyme inhibition.

For determining the spectral curves we will need to obtain a strip-chart recorder for our Beckman DB spectrophotometer since none is presently on hand. Also, we will need to use a high speed ultracentrifuge for preparing the microsomal fractions, which is not available in the Department of Pharmacology, but we have received permission from the Chairman of the Biochemistry Department here to use their Beckman models L-1 and L-2 ultracentrifuges for this purpose.

The Albany area is presently lacking a bovine abbatoir facility that does a sufficient volume of daily slaughtering to provide us with the approximately 50 bovine eyes, necessary to provide enough pigment epithelia to conduct the projected studies, since this structure is

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Leonard Procita

Leonard Procita, Ph.D.
Professor & Acting Chairman
Department of Pharmacology

Checks payable to

Mr. Bernard Siegel
Vice President, Business & Finance

Mailing address for checks

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Principal investigator

Typed Name Alphonse J. Ingenito, Ph.D.

Signature Alphonse J Ingenito Date 12/30/74

Telephone 518--445-5413
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Responsible officer of institution

Typed Name Eugene H. Horn, Ph.D.

Title Associate Dean

Signature [signature] Date 12/30/74

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8. Brief statement of working hypothesis:

The hypotheses to be tested are: (1) that tobacco smoke and CO cause deficits in the function of the visual system by direct actions on the retina, rather than elsewhere in the neural visual pathways and brain; (2) that these effects are not directly due to carboxyhemoglobin (HbCO) formation with loss of blood O_2 carrying function but are due instead to biochemical effects of CO on the retina itself; (3) that a major biochemical effect of tobacco smoke and CO on the retina is on a cytochrome p-450 microsomal electron-transporting oxidase system in the pigment epithelium which might be involved in retinol-retinaldehyde interconversions and (4) that these effects occur at tissue concentrations of CO theoretically attainable in the smoking human.

9. Details of experimental design and procedures (append extra pages as necessary)

The carbon monoxide (CO) content of tobacco smoke has been estimated at between 2 and 6%, depending upon the type of tobacco product and the manner of smoking (Wynder and Hoffmann 1967). Such concentrations, when inhaled by heavy smokers, could conceivably result in blood carboxyhemoglobin (HbCO) levels as high as 10% (van Proosdij 1960). A variety of biological effects of such concentrations, or even lower, have been reported and many of these are said to be detrimental to health (see, eg studies cited by Larson et al., 1961, 1968, 1971; Goldsmith and Landaw, 1968 and Ayres et al., 1969). One biological system especially sensitive to CO is the visual system, and effects on visual acuity and thresholds (Halperin, McFarland et al., 1959 and later studies by the McFarland group; Beard and Grandstaff, 1970) are observed at HbCO levels well within the smoking range, i.e. 5% or even less. The subject of "tobacco amblyopia" is, after a stormy history beginning in the late 1800's, still a debated issue. Although some authorities do not consider this to be a true tobaccogenic disease and believe it to be due, rather, to nutritional deficiencies sometimes associated with heavy tobacco usage, others still maintain that visual defects are a direct consequence of heavy smoking (studies as cited by Grant, 1974; Larson et al., 1961, 1968, 1971 and Shapero 1971). According to some accounts, tobacco amblyopia has the peculiarity of being associated with heavy cigar and pipe smoking and rarely with cigarette smoking (Rucker and Kearns, 1961 as cited by Larson and Sylvette, 1968, pg. 382). Since cigar and pipe smoke are said to contain more CO than cigarette smoke due to incomplete combustion (van Proosdij, 1960, Wynder and Hoffmann 1967), this may account for the latter finding.

The disturbances of visual function often seen following severe CO intoxication are usually attributed to effects on the visual areas of the brain (Grant, 1974, LaPresle and Fardeau, 1967 and other studies in the same volume) but the site(s) of action of CO in the visual system following the milder exposures to CO as with smoking and environmental concentrations, although sometimes ascribed to the brain (Beard and Grandstaff, 1970) are in fact unknown (Halperin et al., 1959). Since the retina is sensitive to toxic agents in a very selective fashion (Potts, 1965, Grant, 1974) it seemed to us to be a likely candidate for some selective biochemical action of CO. The ability of CO to bind to heme pigments such as hemoglobin and the cytochromes is

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9. Details of experimental design and procedures - Continued

but a tiny layer of the retina. However, the Tobin Packing Company, a large pork abbatoir in our area, has agreed to supply us with freshly removed porcine eyes for this purpose. We will therefore adapt all published methods for bovine eyes to studies on porcine eyes instead. We can find no reason at present why the porcine eye should not serve equally well. We will, however, attempt to obtain enough bovine material for some preliminary studies comparing the pigment epithelia of both species.

2. Studies on Incubated Rabbit Retina

The metabolism of rabbit retina has been intensively studied and much is known about its carbohydrate and energy metabolism (Graymore 1969). We have already done some preliminary studies on the effects of CO on glucose uptake and lactate production in the light adapted retina, but the data is too little to detect any pattern of effect, as yet. We plan to continue these studies, using either the Warburg Technique (Cohen and Noell, 1960) or by using the Dubnoff metabolic shaker (Kornblueth et al., 1953, Baeyens et al., 1971) concentrating on ATP and glucose utilization and on lactate and pyruvate production in the incubation media equilibrated with N_2 or CO mixtures or with tobacco smoke or aqueous tobacco smoke extracts. The object here will be to determine whether carbohydrate energy metabolism, in general, is affected by CO or tobacco smoke, so as to provide alternative sites of action for CO other than those we have postulated. It might, e.g., be equally plausible that CO could bind to some cytochrome in the electron-transport chain of retinal visual cell mitochondria and thus diminish ATP production there, eventually disrupting membrane processes and other aspects of energy metabolism as reflected by a decreased

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enzyme systems in pigment epithelium and those describing the in-vitro yes produced by the CO-cytochrome complexes which we hope to find evidence for in our biochemical studies. We have in the past, tried to do these spectral curves manually and find this to be a very time-consuming and inaccurate alternative.

The semi-micro Waring Blendor is needed for making homogenates of the bovine and porcine pigment epithelium.

In summary we have tried, especially with the requests for permanent equipment, to keep our budget in line with our immediate requirements for this project and we will, whenever possible, use our existing equipment, and will borrow from others in the institution where feasible, as, eg in our need for a high-speed centrifuge.

another laboratory but we were obliged to return it to its original owner recently.

The miscellaneous items of electronic and gas delivery equipment

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9. Details of experimental design and procedures - Continued

photostimulator which will need to be purchased. A Tektronix Dual Beam Oscilloscope with Polaroid Camera or a Beckman Dynograph will be used to record the electrical potentials involved.

2. Isolated Perfused Cat Eye (Hemoglobin-Free Perfusate):

The above intact cat preparation, although important to establish the effects of CO and tobacco smoke on the relatively intact eye, presents some problems in interpretation because the effects of HbCO, however slight at the low levels employed, complicate interpretations somewhat. To establish the possibility of direct effects of CO on the retina, and to exclude the possibility of HbCO-induced hypoxia, it will be necessary to assess retinal function in a hemoglobin-free system. The isolated perfused cat eye preparation of Gouras and Hoff (1970) offers a unique approach since the ERG may be recorded in the intact eye after removal from the animal and perfusion in a bath with a hemoglobin-free physiological salt solution under controlled conditions. The optics of this system are rather complex and will need simplification in our approach to its construction. The perfusion system (via the ophthalmociliary artery) is rather simple and should be adaptable to our existing perfusion equipment with the exception of the perfusion chamber which we will need to have constructed. The preparation is said to maintain normal ERG configurations for many hours; well within the time period necessary for us to demonstrate effects of CO and tobacco smoke. The perfusing solution will be bubbled with various concentrations of CO (or tobacco smoke) and the effects again compared to equivalent degrees of nitrogen hypoxia.

3. Isolated Retina-Optic Nerve Preparation (Rabbit Eye):

The previously described perfused cat eye preparation of Gouras and Hoff will provide some insight as to CO effects on the retina in

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enzyme systems in pigment epithelium and those describing the in-vitro retina-optic nerve preparation mention the necessity of using glass distilled water since trace heavy metals (e.g. copper) found in water obtained from our copper Barnstead stills might cause enzyme inhibition and inhibit the function of the retina in these preparations. We do not presently have a source of glass-distilled water and the $600 listed for this purpose is for the smallest and least expensive model we can find to provide us with working quantities.

The Grass photostimulator is needed for the ERG studies on the anesthetized cat and for the retina-optic nerve preparation.

The Dubnoff metabolic shaker is needed as the incubator for the in-vitro metabolic studies in porcine retinal pigment cell suspensions and on isolated rabbit retinae. We recently did some preliminary studies with rabbit retinae using a metabolic shaker borrowed from another laboratory but we were obliged to return it to its original owner recently.

The miscellaneous items of electronic and gas delivery equipment are for ERG and nerve electrodes, the possible neeed for a closed system of respiratory valves for our anesthetized cat preparation.

The Lucite chambers for the isolated rabbit retina-optic nerve and the perfused cat eye preparation will need to be made to order either from our own shop or from an outside source since these are not commercially available. The $500 allowed for this also allows a bit for specialized tubing, valves and reservoirs which may be needed as well.

The Strip-Chart recorder for the Beckman Model DB spectro-photometer is essential for the accurate determination of the spectral cur-

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14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
A. J. Ingenito, Ph.D.	50	$ 5,000
Technical		
To be recruited	100	$ 9,000
Sub-Total for A		$14,000
B. Consumable supplies (by major categories)		
Reagents, biochemicals, gases, etc.		$ 2,000
Glassware, routine		$ 1,000
Perfusion & chromatography apparatus, glassware		$ 1,000
Surgical instruments & supplies		$ 500
Sub-Total for B		$ 4,500
C. Other expenses (itemize)		
Animals, cats, and rabbits and animal care		$ 2,000
Bovine or porcine eyes from local abbatoir		750
Photographic & illustration work for manuscript preparation		400
Computer analyses of data		100
Travel to meetings		500
Sub-Total for C		$ 3,750
Running Total of A + B + C		$22,250
D. Permanent equipment (itemize)		
Glass distilled water apparatus		600
Grass Photostimulater		550
Dubnoff Metabolic Shaker		850
Misc. items of electronic & gas delivery equipment		750
Lucite chambers for retina and eye perfusion		500
Strip-chart recorder for spectrophotometer		950
Semi-micro Waring Blendor		120
Sub-Total for D		$ 4,320
E. Indirect costs (15% of A+B+C)	E	$ 3,340
	Total request	$29,910

O.K. L.B.

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	14,500	4,000	3,800	1,000	3,340	26,640
Year 3	--	--	--	--	--	--

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The principal investigator has two laboratories and an office presently assigned to him totaling about 650 sq. ft. of floor space with the option of an additional laboratory of about 150 sq. ft. for studies in a dark environment, should the grant be approved and funded. The laboratories are well equipped with routine glassware, chemicals, refrigerators, water, electricity and vacuum lines and hoods for venting noxious gases. Equipment on hand necessary for these studies include a Beckman DB Spectrophotometer, Lourdes and International refrigerated centrifuges and rotors, Gilson Differential Respirometer, Beckman Offner Type R Dynograph with appropriate couplers and preamplifers, Tectronix Dual-Beam Oscilloscope, Polaroid oscilloscope camera, Beckman Blood pH meter, Radiometer pH meter, Instrumentation Laboratories Model 182 CO-Oximeter, Radimeter blood-gas monitor and electrodes, various gas handling valves and mixing equipment, two Tamson constant - temperature water baths, various glass reservoirs and other items needed for extracorporeal perfusions, and two Barnstead copper water stills for distilled water. The College's accredited animal facilities occupy the entire basement of two buildings in the Medical College Complex and will provide expert care and handling of animals. There is also a computer facility available for statistical analyses and the Department of Pharmacology has a small Hewlett-Packard Computer programmed for routine statistical analyses.

11. Additional facilities required:

As stated above an ultracentrifuge providing forces of 100,000 x g is required for the studies on pigment epithelium microsomal fractions. The cost of such an instrument with appropriate rotors is approximately $12,000, a price we feel to be disproportionate to the relative necessity of it for the proposed study. We have obtained permission to use an ultracentrifuge for that aspect of the study from the Chairman of the Department of Biochemistry where such a centrifuge is located.

12. Biographical sketches of investigator(s) and other professional personnel (append):

A curriculum vitae is attached.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available):

See attached curriculum vitae, two papers and five abstracts.

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